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AU Muhlhauser J; Merrill M J; Pili R; Maeda H; Bacic M; Bewig B; Passaniti A; Edwards N A; Crystal R G; Capogrossi M C
CS Pulmonary Branch, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, MD, USA.
SO CIRCULATION RESEARCH, (1995 Dec) 77 (6) 1077-86.

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TI Liposome-mediated BDNF cDNA transfer in intact and injured rat brain.
AU Iwamoto Y; Yang K; Clifton G L; Hayes R L
CS Department of Neurosurgery, University of Texas Houston Health Science Center, Houston 77030, USA.
NC PO1 NS31998 (NINDS)
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SO NEUROREPORT, (1996 Jan 31) 7 (2) 609-12.

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GA The Genuine Article (R) Number: ZM121
TI Responses of young and aged rat CNS to partial cholinergic immunolesions and NGF treatment
AU Wortwein G; Yu J; ToliverKinsky T; PerezPolo J R (Reprint)
CS UNIV TEXAS, MED BRANCH, DEPT HUMAN BIOL CHEM & GENET, GALVESTON, TX 77555 (Reprint); UNIV TEXAS, MED BRANCH, DEPT HUMAN BIOL CHEM & GENET, GALVESTON, TX 77555; RIGSHOSP, LAB NEUROPSYCHIAT, DK-2100 COPENHAGEN, DENMARK
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SO JOURNAL OF NEUROSCIENCE RESEARCH, (1 MAY 1998) Vol. 52, No. 3, pp. 322-333.

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GA The Genuine Article (R) Number: 164UH
TI Nerve growth factor expressed in the medial septum following in vivo gene delivery using a recombinant adeno-associated viral vector protects cholinergic neurons from fimbria-fornix lesion-induced degeneration
AU Mandel R J (Reprint); Gage F H; Clevenger D G; Spratt S K; Snyder R O; Leff S E
CS LUND UNIV, WALLENBERG NEUROSCI CTR, NEUROBIOL SECT, SOLVEGATAN 17, S-22362 LUND, SWEDEN (Reprint); CELL GENESYS INC, DEPT PRECLIN BIOL, FOSTER CITY, CA 94404; SALK INST BIOL STUDIES, GENET LAB, LA JOLLA, CA 92037
CYA SWEDEN; USA
SO EXPERIMENTAL NEUROLOGY, (JAN 1999) Vol. 155, No. 1, pp. 59-64.

Shin-Lin Chen

$VEGF_{165}$ Expressed by a Replication-Deficient Recombinant Adenovirus Vector Induces Angiogenesis In Vivo

Judith Mühlhauser, Marsha J. Merrill, Roberto Pili, Hiroyuki Maeda, Mima Bacic, Burkhard Bewig, Antonino Passaniti, Nancy A. Edwards, Ronald G. Crystal, Maurizio C. Capogrossi

Abstract To evaluate the concept that localized delivery of angiogenic factors via virus-mediated gene transfer may be useful in the treatment of ischemic disorders, the replication-deficient adenovirus (Ad) vector AdCMV.$VEGF_{165}$ (where CMV is cytomegalovirus and VEGF is vascular endothelial growth factor) containing the cDNA for human $VEGF_{165}$, a secreted endothelial cell–specific angiogenic growth factor, was constructed. Human umbilical vein endothelial cells (HUVECs) and rat aorta smooth muscle cells (RASMCs) infected with AdCMV.$VEGF_{165}$ (5 and 20 plaque-forming units [pfu] per cell) demonstrated VEGF mRNA expression and protein secretion into the supernatant. Furthermore, the conditioned medium from these cells enhanced vascular permeability in vivo. In contrast, neither VEGF mRNA nor secreted protein was found in uninfected HUVECs or RASMCs or in cells infected with the control vector AdCMV.βgal (where βgal is β-galactosidase). Assessment of starved HUVECs at 14 days demonstrated sixfold more cells for AdCMV.$VEGF_{165}$-infected HUVECs (20 pfu per cell) than for either infected or uninfected control cells. RASMC proliferation was unaffected by infection with AdCMV.$VEGF_{165}$. When plated in 2% serum on dishes precoated with reconstituted basement membrane (Matrigel), HUVECs infected with AdCMV.$VEGF_{165}$ (20 pfu per cell) differentiated into capillary-like structures. Under similar conditions, both uninfected HUVECs and HUVECs infected with AdCMV.βgal did not differentiate. To evaluate the ability of AdCMV.$VEGF_{165}$ to function in vivo, either AdCMV.$VEGF_{165}$ or AdCMV.βgal (2×10^{10} pfu) was resuspended in 0.5 mL Matrigel and injected subcutaneously into mice. Immunohistochemical staining demonstrated VEGF in the tissues surrounding the Matrigel plugs containing AdCMV.$VEGF_{165}$ up to 3 weeks after injection, whereas no VEGF was found in the control plugs with AdCMV.βgal. Two weeks after injection, there was histological evidence of neovascularization in the tissues surrounding the Matrigel containing AdCMV.$VEGF_{165}$, whereas no significant angiogenesis was observed in response to AdCMV.βgal. Furthermore, the Matrigel plugs with AdCMV.$VEGF_{165}$ demonstrated hemoglobin content fourfold higher than the plugs with AdCMV.βgal. Together, these in vitro and in vivo studies are consistent with the concept that Ad vectors may provide a useful strategy for efficient local delivery of $VEGF_{165}$ in the treatment of ischemic diseases. (*Circ Res.* 1995;77:1077-1086.)

Key Words • angiogenesis • endothelium • gene therapy • VEGF • vascular permeability factor

The treatment of ischemic disorders due to arterial occlusion relies on surgical revascularization or angioplasty. The size of the artery involved, the complexity of the arterial lesions that cause the occlusion, and the general clinical conditions of the patient frequently prevent revascularization of the ischemic tissues. Therefore, less invasive approaches need to be developed in order to treat patients who are not candidates for either surgery or angioplasty. The development of collateral circulation is known to improve blood flow to ischemic tissues and to alleviate the symptoms due to ischemia.[1,2] Several growth factors have been shown to induce neovascularization,[3,4] and gene transfer of angiogenic factors may provide a novel approach to enhance collateral blood flow and to relieve ischemia. Recently, replication-deficient recombinant Ad vectors have been used for gene transfer studies[5,6] and appear to have several attractive properties.[7,8] Ad vectors can transduce a variety of tissues, including endothelium,[9] myocardium,[10-13] and skeletal muscle cells.[13] Moreover, Ad vectors appear safe for clinical use, and there are ongoing clinical trials with patients with cystic fibrosis.[14] Thus, Ad vectors may be used to transfer the cDNA for angiogenic polypeptides into ischemic tissues. To this end, a vector that carries the cDNA for VEGF was engineered. Endogenous VEGF may have a role in the angiogenic response that occurs during ischemia, since endogenous VEGF mRNA increases in hypoxic cells in vitro and in glioblastoma cells in vivo near necrotic areas, which are

Received February 3, 1995; accepted August 21, 1995.

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Previously presented as preliminary results in abstract form (*J Cell Biochem.* 1994;18A:DZ315).

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Selected Abbreviations and Acronyms

βgal = β-galactosidase
Ad = adenovirus
CMV = cytomegalovirus
HUVEC = human umbilical vein endothelial cell
KLH = keyhole limpet hemocyanin
pfu = plaque-forming units
RASMC = rat aortic smooth muscle cell
VEGF = vascular endothelial growth factor
X-gal = 5-bromo-4-chloro-3-indolyl-β,D-galactopyranoside

presumably hypoxic.[15-17] VEGF is a heparin-binding glycoprotein also known as vascular permeability factor. VEGF is produced by a variety of tissues, including vascular smooth muscle cells[18,19]; VEGF binding sites are present on endothelial cells, and the mitogenic action of VEGF, unlike that of other growth factors, is selective for endothelial cells.[20-22] Alternative splicing of the human VEGF gene transcript produces four mRNA forms that code for polypeptides of 206, 189, 165, and 121 amino acids; the 165- and 121-amino acid forms are readily secreted, but those with 206 and 189 amino acids remain cell-associated.[23,24] The human $VEGF_{165}$ form was used in the present study, since this form has been shown to be angiogenic[25] and to be readily diffusible after secretion. The study was designed to determine whether endothelial and vascular smooth muscle cells infected with the Ad vector that codes for $VEGF_{165}$ produce a functional protein and whether by this approach it is possible to induce endothelial cell differentiation and/or proliferation in vitro and angiogenesis in vivo.

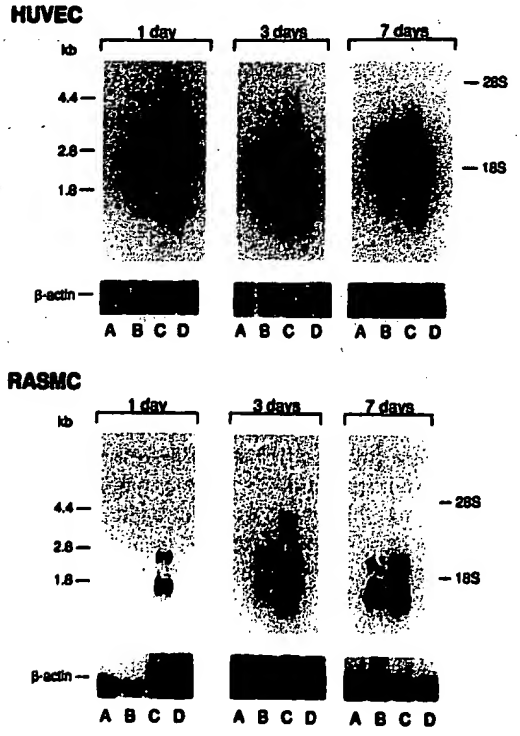

FIG 1. Expression of $VEGF_{165}$ mRNA in HUVECs and RASMCs. One day before the beginning of the experiment, cells were infected for 24 hours (see "Materials and Methods") either with AdCMV.$VEGF_{165}$ or with AdCMV.βgal, and expression of $VEGF_{165}$ or control actin mRNA was assessed 1, 3, and 7 days later. Lanes are as follows: A, uninfected cells; B, cells infected with AdCMV.$VEGF_{165}$ (5 pfu per cell); C, cells infected with AdCMV.$VEGF_{165}$ (20 pfu per cell); and D, cells infected with AdCMV.βgal (20 pfu per cell).

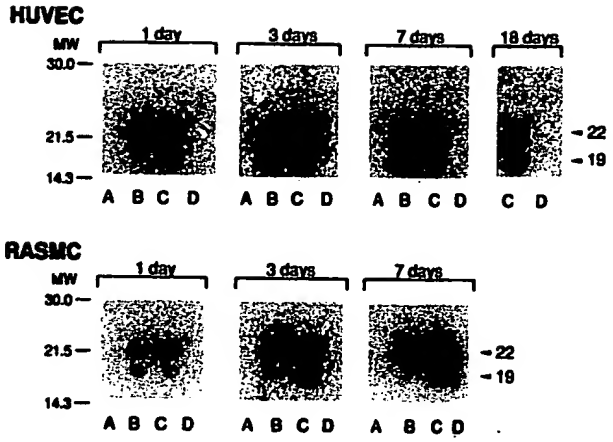

FIG 2. Expression of $VEGF_{165}$ protein by HUVECs and RASMCs. Conditioned medium from HUVEC and RASMC cultures infected as in Fig 1 was examined for the presence of $VEGF_{165}$ protein at different times after infection. In all dishes, the medium was changed 24 hours before the collection of the samples. Lanes are as follows: A, uninfected cells; B, cells infected with AdCMV.$VEGF_{165}$ (5 pfu per cell); C, cells infected with AdCMV.$VEGF_{165}$ (20 pfu per cell); and D, cells infected with AdCMV.βgal (20 pfu per cell).

Materials and Methods

Ad Vectors

The replication-deficient recombinant Ad vector containing the cDNA for $VEGF_{165}$ was engineered according to a technique previously described.[26] Briefly, the cDNA for $VEGF_{165}$, including the signal sequence for secretion,[27] was inserted into an expression plasmid[26] and was under the control of the constitutive CMV immediate-early promoter/enhancer. The expression plasmid also contained the Ad 5 sequence from nucleotide 3384 to nucleotide 5778 (9.24 to 16.05 map units), which served as the homologous recombination sequence. The plasmid carrying the cDNA for $VEGF_{165}$ was cotransfected with the plasmid pJM17 (from F. Graham, McMaster University, Hamilton, Ontario, Canada) into 293 cells (American Type Culture Collection, CRL1573). The plasmid pJM17 contains the full-length Ad 5 DNA (36 kb) and pBRX, a 4.3-kb insert placed in the E1 region, thus exceeding by ≈2 kb the maximum packaging limit of DNA into the Ad capsid.[28] Homologous recombination between the expression plasmid and pJM17 in 293 cells replaced the E1 region and pBRX insert with the expression cassette from the expression plasmid. The growth of these E1-deleted Ads is limited to 293 cells, a human embryonic kidney cell line that has been transformed by Ad 5 and expresses the E1 region in trans. Culture medium for the 293 cells was improved minimal essential medium with 10% heat-inactivated fetal bovine serum, 2 mmol/L glutamine, 50 U/mL penicillin, and 50 μg/mL streptomycin (all from Biofluids). After cotransfection, individual viral plaques were isolated and amplified in 293 cells. The control vector was AdCMV.βgal, which carries the cDNA for the *Escherichia coli* lacZ gene and codes for the enzyme β-galactosidase.[29] AdCMV.$VEGF_{165}$ and AdCMV.βgal were propagated in 293 cells and were purified by CsCl density purification. Subsequently, the preparations were dialyzed and stored in the dialysis buffer (10 mmol/L Tris-HCl and 1 mmol/L $MgCl_2$, pH 7.4) with 10% glycerol at −70°C. The titer of each viral stock was determined by plaque assay in 293 cells as previously

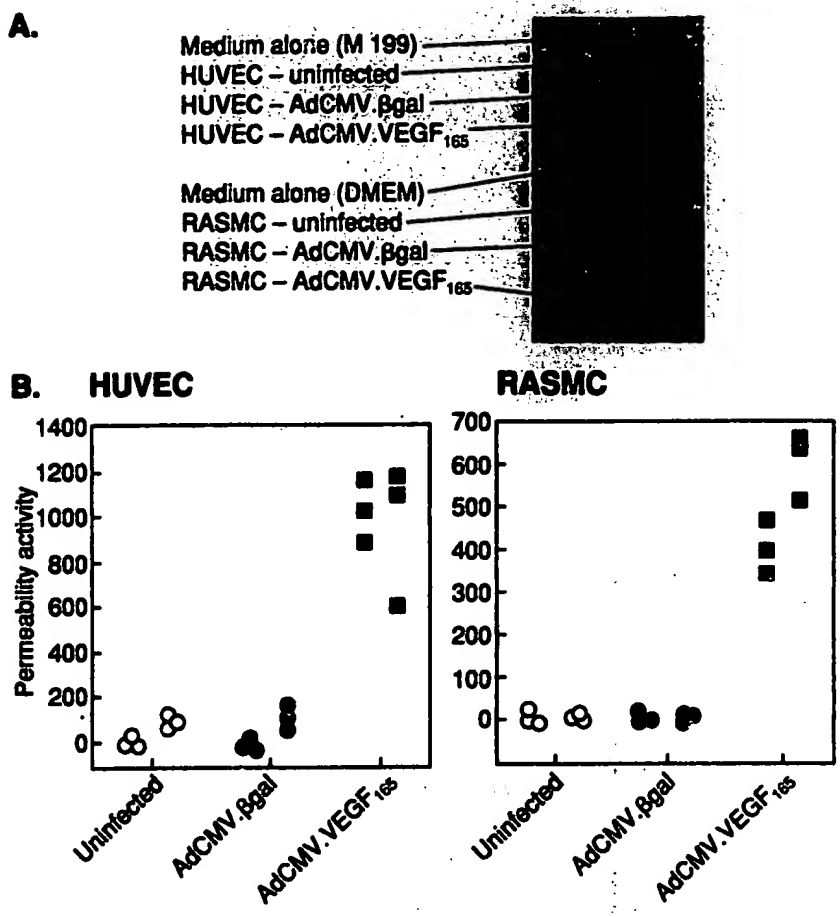

Fig 3. Effect of conditioned medium from HUVECs and RASMCs on vascular permeability as assessed by the Miles assay. A, Medium from both HUVECs and RASMCs infected with AdCMV.VEGF$_{165}$ caused extravasation of Evans blue dye. In contrast, there was no significant increase in permeability with the injection of conditioned medium from uninfected and AdCMV.βgal-infected cells or medium that had not been in contact with cells. B, Quantitative assessment of permeability activity of conditioned medium is expressed as the $A_{620}\times10^3$ (see "Materials and Methods"). Results represent six determinations from two independent experiments. The increase in permeability due to the conditioned medium from either HUVECs or RASMCs infected with AdCMV.VEGF$_{165}$ was significantly higher than for either control group ($P<.0001$ for AdCMV.VEGF$_{165}$ vs either uninfected or AdCMV.βgal-infected HUVECs and RASMCs).

described,[6] and the titers consistently ranged between 5×10^9 and 2×10^{11} pfu/mL.

mRNA Isolation and Northern Blot Analysis

HUVECs and RASMCs infected for 24 hours either with AdCMV.VEGF$_{165}$ (5 or 20 pfu per cell) or with AdCMV.βgal (20 pfu per cell) were examined for the presence of VEGF mRNA at 1, 3, and 7 days after completing the infection. RNA was isolated according to the method of Chomczynski and Sacchi.[30] After isolation, RNA was subjected to electrophoresis and transferred to nylon membranes.[31] For use as a probe in Northern blot analysis, VEGF$_{165}$ insert was labeled in a random primer extension reaction and hybridized by using Stratagene Quick Hybrid Solution according to manufacturer's instructions.

Western Blot Analysis

HUVECs and RASMCs were infected as described above. For these experiments, the dishes were washed, and fresh medium was added 24 hours before the sample collection for Western analysis. This approach allowed us to examine the production of VEGF$_{165}$ over the course of 24 hours at different time points after the infection. Polyclonal antibodies to the first 20 amino acids of mature human VEGF N-terminus were prepared as previously described,[32] except the peptide was conjugated to a carrier protein, KLH, by 0.2% glutaraldehyde. Aliquots of conditioned medium were separated on a 12.5% polyacrylamide gel under reducing conditions and transferred to nitrocellulose (Schleicher & Schuell). Membranes were processed by using ECL detection reagents according to manufacturer's instructions (Amersham). Anti-VEGF antiserum was used at 1:500 dilution. Secondary antibody (donkey anti-rabbit IgG, horseradish peroxidase–conjugated, Jackson Research) was used at 1:5000 dilution.

VEGF Enzyme-Linked Immunosorbent Assay

Enzyme immunoassay for the detection of human VEGF was carried out with Cytokit Red VEGF (CytImmune Sciences, Inc). Supernatants of HUVECs infected with either AdCMV.VEGF$_{165}$ or AdCMV.βgal (20 pfu per cell) were processed 1, 3, 7, and 17 days after infection. The medium in each dish was changed 24 hours before the collection of the supernatant. The assay procedure was carried out according to the supplier's instructions, and absorbance at 490 nm was determined on a plate reader. VEGF concentration was normalized to cell number.

Vascular Permeability Assay

Conditioned medium obtained from HUVEC and RASMC cultures 3 days after infection with AdCMV.VEGF$_{165}$ (20 pfu per cell) was tested in guinea pigs for its permeability activity in a modified Miles assay.[33] The medium contained 2% serum, and its effect was compared with the conditioned medium from uninfected cells or from cells infected with AdCMV.βgal (20 pfu per cell). The Evans blue dye was eluted from skin punches in formamide and quantified at A_{620} as previously described.[33] For the quantitative determination of the permeability changes, the A_{620} values from either medium 199 (Biofluids) or DMEM (Biofluids) alone, which had not been previously in contact with cells, were subtracted from the values obtained with conditioned medium from uninfected cells and from both AdCMV.βgal- and AdCMV.VEGF$_{165}$-infected cells.

Endothelial and Vascular Smooth Muscle Cell Proliferation

HUVECs and RASMCs (passages 5 to 10) were used for this study. HUVECs (Advanced Biotechnology, Inc) were cultured in medium 199 supplemented with 20% calf serum (Hyclone Inc) and 100 μg/mL endothelial cell growth supplement (Col-

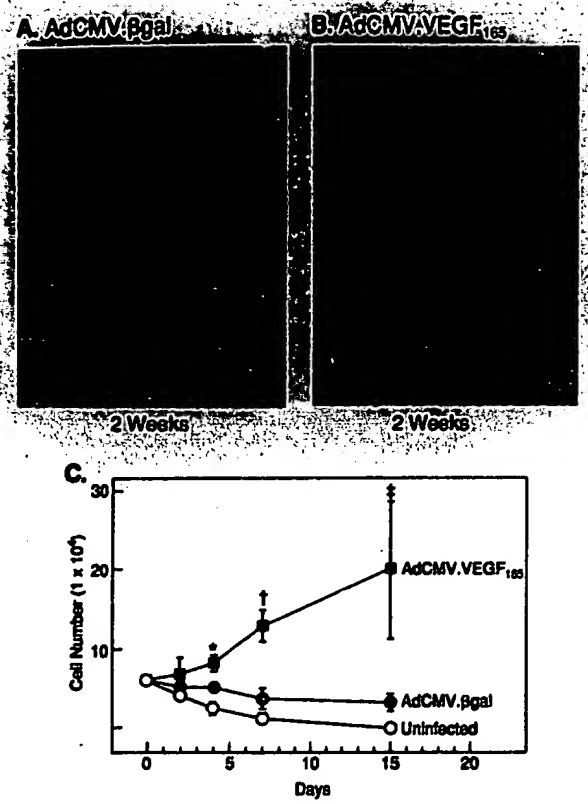

FIG 4. Effect of AdCMV.VEGF$_{165}$ infection on proliferation of HUVECs. Endothelial cells infected (20 pfu per cell) with AdCMV.VEGF$_{165}$ or with AdCMV.βgal and uninfected control cells were cultured for 2 weeks (see "Materials and Methods"). A, Representative example of cells exposed to AdCMV.βgal. B, Representative example of cells exposed to AdCMV.VEGF$_{165}$. Panels A and B show cells 2 weeks after infection. C, Average HUVEC number at different times after infection. The results represent the average of three experiments. *P* values for each time point refer to AdCMV.VEGF$_{165}$ vs either control (*P<.01; †P<.005; ‡P<.05).

laborative Research Inc). RASMCs isolated as previously described[34] were grown in DMEM supplemented with 10% fetal bovine serum (Biofluids). For the growth assay, 5×10^4 cells were seeded in 35-mm Petri dishes in standard growth medium. Before infection, the growth medium in the dishes was substituted with medium without growth supplement and with 2% serum. After 24 hours, cells were infected with 5 or 20 pfu per cell AdCMV.VEGF$_{165}$ or AdCMV.βgal; a third group of cells was not infected. Exposure to the Ad vector lasted 24 hours; after which, the medium was removed and substituted either with medium 199 with 10% calf serum (HUVECs) or with DMEM with 2% fetal bovine serum (RASMCs). The medium in all dishes was changed every other day, and cells were harvested at 48 hours, 96 hours, 1 week, and 2 weeks by trypsin/EDTA (Biofluids) treatment. Cell counts were performed in triplicate by using a Coulter counter (Coulter Corp).

Endothelial Cell Differentiation In Vitro

Endothelial cells plated on plastic in the presence of mitogens and serum proliferate and form a confluent cobblestone monolayer. In contrast, endothelial cells plated onto a gel of basement membrane proteins (Matrigel) exhibit a low rate of DNA synthesis, a high rate of migration and invasion of extracellular matrix, and differentiation into multicellular capillary-like structures.[35] However, in low-serum conditions and in the absence of growth factors, endothelial cells on Matrigel do not differentiate into a network of capillary-like structures. HUVECs in serum-free MCDB131 medium (Clonetics) and without growth supplements were infected either with AdCMV.VEGF$_{165}$ or with AdCMV.βgal (20 pfu per cell) 48 hours before trypsinization and replating. Exposure to the Ad vector lasted 24 hours. Another group of cells was not infected and was used as a second control. HUVECs were harvested 48 hours after the infection with trypsin/EDTA and plated in 16-mm wells (8×10^4 cells per well) previously coated with reconstituted basement membrane (Matrigel, 0.3 mL per well, 10 mg/mL) for 1 hour at 37°C, as previously described.[35] After 24 hours, the cells were fixed in PBS-buffered 10% formalin containing 2.5% glutaraldehyde. Capillary-like structures formed by HUVECs were visualized with an inverted microscope (Diaphot), photographed with a Polaroid camera, and quantified by optical imaging (IMAGE-1 analysis system, Uni-

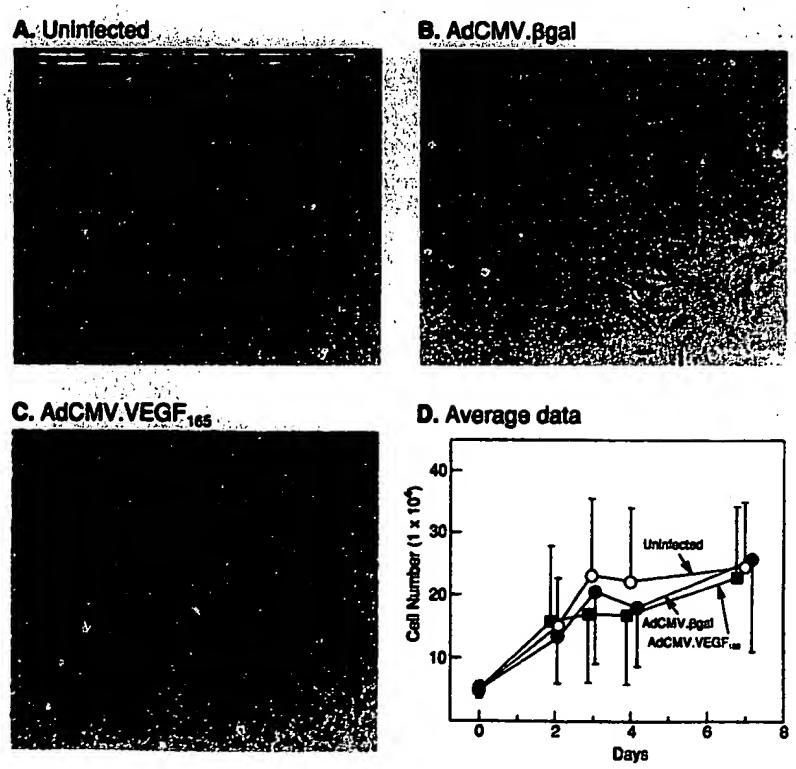

FIG 5. Effect of AdCMV.VEGF$_{165}$ infection on proliferation of RASMCs. Smooth muscle cells infected (20 pfu per cell) either with AdCMV.VEGF$_{165}$ or with AdCMV.βgal and uninfected control cells were cultured for 1 week (see "Materials and Methods"). A, Representative example of uninfected control cells. B, Representative example of RASMCs infected with AdCMV.βgal. C, Representative example of cells infected with AdCMV.VEGF$_{165}$. Panels A through C show cells at 1 week after infection. D, Average RASMC number at different times after infection. Results represent the average of three experiments. There is no significant difference among groups.

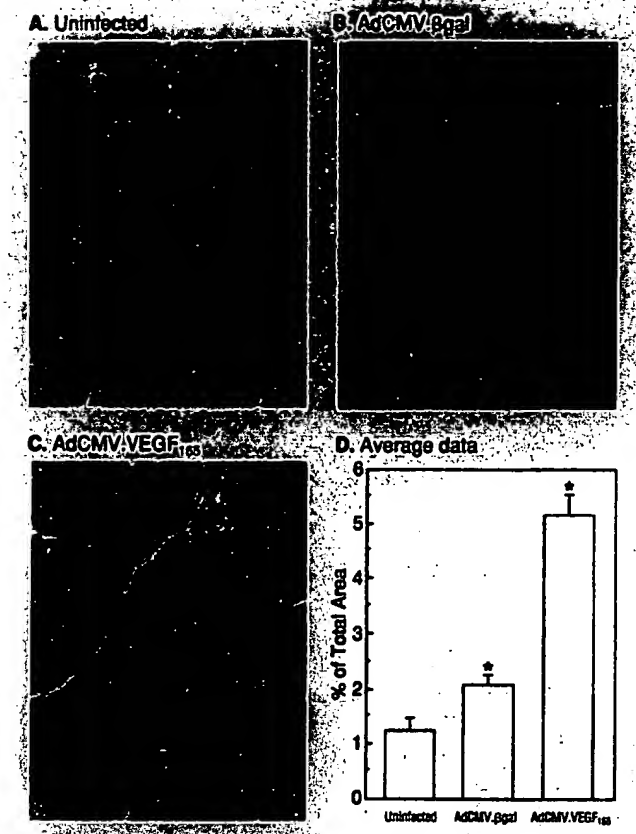

FIG 6. Effect of AdCMV.VEGF$_{165}$ infection on differentiation of HUVECs. Endothelial cells infected (20 pfu per cell) either with AdCMV.VEGF$_{165}$ or with AdCMV.βgal and uninfected control cells were plated on plastic dishes precoated with Matrigel and grown in the absence of serum for 24 hours. Panels show representative examples 1 day after the beginning of the experiment. A, Uninfected control cells. B, Control cells infected with AdCMV.βgal. C, Cells infected with AdCMV.VEGF$_{165}$. These cells elongated, established connections with each other, and formed a capillary-like network. In contrast, most uninfected and AdCMV.βgal-infected HUVECs shown in panels A and B preserved a round appearance. D, Quantitative assessment of the percent dish area occupied by endothelial cells and by capillary-like structures. Infection with AdCMV.VEGF$_{165}$ caused a significant increase in the capillary network area above both control groups, and infection with AdCMV.βgal enhanced the capillary network above uninfected control cells (*P<.0001 vs either of the other two groups). Results represent the average of three experiments.

versal Imaging Corp). The surface area occupied by the endothelial cells and by the capillary network was measured in eight optical fields for three wells. The percentage of the area from triplicate wells was averaged, and the results were expressed as the mean±SD from three experiments.

Ad-Mediated Gene Transfer In Vivo

In order to assess the effects of Ad-mediated gene transfer in vivo, either AdCMV.VEGF$_{165}$ or AdCMV.βgal (2×10^{10} pfu) was resuspended in 0.5 mL Matrigel. Subsequently, C57BL mice (Jackson Laboratories, Bar Harbor, Me) were injected subcutaneously, near the abdominal midline, with 0.5 mL Matrigel containing either AdCMV.VEGF$_{165}$ or AdCMV.βgal. Additional animals were injected with uninfected Matrigel. Mice were studied according to four different protocols: (1) To establish whether Ad vectors resuspended in Matrigel infect the surrounding tissues, mice were injected either with Matrigel containing AdCMV.βgal (n=5) or Matrigel alone (n=3). The animals were killed 6 days after injection, and the Matrigel plugs were removed and fixed as described above for endothelial cells. Subsequently, the Matrigel plugs were sectioned, stained with X-gal as previously described,[29] and examined for evidence of blue staining. (2) To establish the duration of transgene expression in vivo, mice were injected either with Matrigel containing AdCMV.VEGF$_{165}$ (n=9), AdCMV.βgal (n=9), or Matrigel alone (n=9). Animals were killed, and the Matrigel plugs were removed 3, 7, and 21 days after injection. Tissue blocks were immersed in OCT compound (Miles Inc) and rapidly frozen in liquid nitrogen. Tissue blocks were stored at −70° for <1 month. For immunohistochemical evaluation, 10-μm frozen sections (Microm cryotome) were mounted on silanated slides (Digene Diagnostics). Sections were air-dried for 15 minutes, and either stored at −70° for up to 48 hours or fixed immediately in 1× Histochoice (Amresco) containing 0.1% Triton X-100 (Sigma Chemical Co) for 12 minutes. After they were washed with PBS (pH 7.4), slides were incubated in 0.5% hydrogen peroxide in methanol to inhibit endogenous peroxidase activity. Anti-VEGF primary rabbit antibodies (see below) were detected by using biotinylated goat anti-rabbit IgG secondary antibody and the avidin-biotin complex and visualized by diaminobenzidine (all detection reagents were from Vector Laboratories). Procedures were performed according to package directions, except sections were kept in blocking solution for at least 45 minutes before the addition of the primary antibody, and incubations with anti-VEGF or control serum (1:6000 dilution) were performed overnight at 4°C. Sections were counterstained in hematoxylin. Anti-VEGF antibodies were produced in rabbits as previously described,[32] except the peptide was conjugated to a carrier protein, KLH, by 0.2% glutaraldehyde. Antibodies to KLH alone were also raised and used as a negative control. Antibody specificity was determined by recognizing human VEGF on Western blots, and both anti-KLH and prebleed serum were used as negative controls to determine background staining. (3) The presence of newly formed blood vessels was evaluated as previously described[36] in mice killed 14 days after the injection of the Matrigel (n=8 mice for each Ad vector; 4 mice were used in each of two separate experiments). The gels were recovered by dissection and fixed. Histological sections were stained with Masson's trichrome stain and evaluated for the presence of neovascularization. The thickness of the stroma surrounding the Matrigel was assessed by measuring the distance between

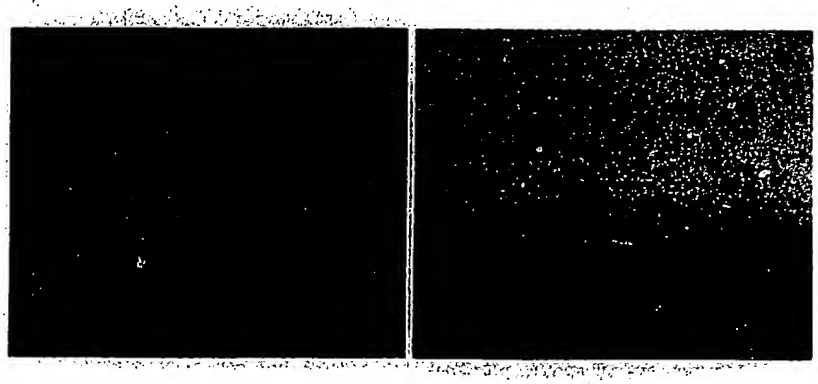

FIG 7. AdCMV.βgal resuspended in Matrigel infects the surrounding tissues in vivo. Arrows show the stroma between the Matrigel plug (mp) and the abdominal muscle (m). A, X-gal–positive cells (blue cells) are present in the stroma surrounding the Matrigel 6 days after coinjection of Matrigel with AdCMV.βgal. B, No X-gal–positive cells are observed after injection of Matrigel alone. It is noteworthy that AdCMV.βgal caused thickening of the stroma. Tissue samples were stained with X-gal (original magnification ×50).

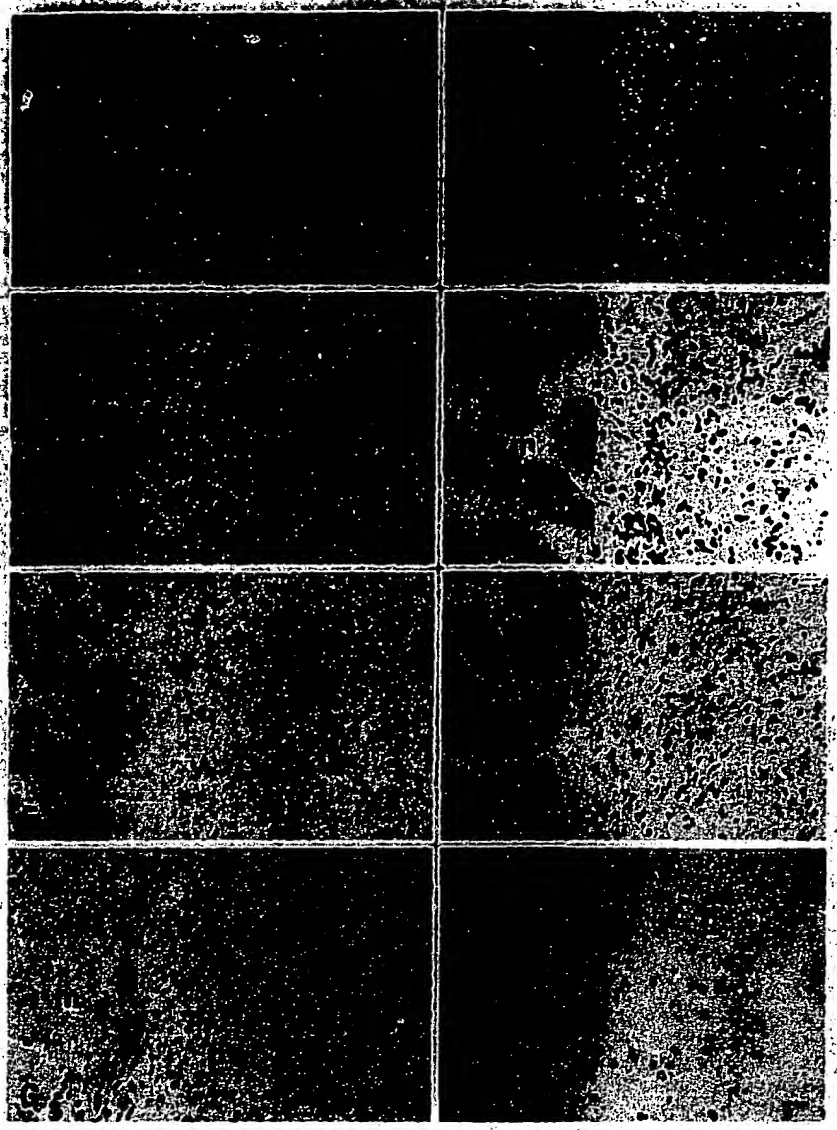

Fig 8. Expression of human VEGF in the tissue surrounding the Matrigel coinjected either with AdCMV.VEGF$_{165}$ (A, C, E, G, and H) or with AdCMV.βgal (B, D, and F). m indicates abdominal muscles. Specimens were analyzed 3 days (A, B, and H), 7 days (C, D, and G), and 21 days (E and F) after injection. VEGF expression is visualized by the brown color in the cells indicated by the arrowheads. VEGF-positive cells are present at 3 days (A), 7 days (C), and 21 days (E) in the tissue surrounding the Matrigel with AdCMV.VEGF$_{165}$, and the intensity of the response is most marked at 7 days (C). In contrast, no VEGF-positive cells are identified in the tissue surrounding the Matrigel with AdCMV.βgal (B, D, and F). When the specimen shown in panel C was incubated in the absence of the primary antibody, no immunoreactivity was observed (G). When the anti-carrier protein antibody was used as the primary antibody on the specimen shown in panel A, only the muscles acquired a brown stain (H). Original magnification ×200.

the surface of the Matrigel and the abdominal muscle in two different histological sections from each plug. Ten measurements were obtained at 50- to 100-μm intervals from each histological section, and the 20 measurements from the two sections were averaged to express stromal thickness for each individual plug. (4) The angiogenic response was quantified by the hemoglobin content of the Matrigel plugs[36] (n=10 mice for each Ad vector; 3 or 4 mice were used in each of three separate experiments).

Statistical Analysis

The results are presented as mean±SD. Statistical analysis was performed by unpaired Student's *t* test, and a value of $P \leq .05$ was taken to indicate statistical significance.

Results

VEGF Expression in Cells Infected With AdCMV.VEGF$_{165}$

Both HUVECs and RASMCs infected with AdCMV.VEGF$_{165}$ produced VEGF$_{165}$ transcripts (Fig 1). The quantity of mRNA produced was higher after infection with 20 than with 5 pfu per cell, and exogenous gene expression persisted for the 1-week duration of this experiment. It is also apparent that two different VEGF transcripts were present and that both transcripts were smaller than the native VEGF$_{165}$ mRNA, which is ≈4 kb.[18,31] This is not surprising because the 5′ and 3′ untranslated regions and the polyadenylation signal of the VEGF$_{165}$ molecule may be significantly longer in the case of native VEGF$_{165}$ than when our expression cassette was used for mRNA expression.

Western analyses of the conditioned medium from HUVECs and RASMCs infected with AdCMV.VEGF$_{165}$ showed that VEGF protein was produced and secreted (Fig 2). As in the case of the mRNA, the amount of VEGF$_{165}$ protein produced was higher after infection with 20 than with 5 pfu per cell, and the VEGF$_{165}$ content in the conditioned medium, as determined by the intensity of the bands in the Western blot, was relatively constant up to 1 week. In one experiment, we examined the VEGF$_{165}$ produced for 2½ weeks after the infection, and we found that VEGF$_{165}$ production persisted throughout the course of the experiment up to 18 days. VEGF$_{165}$ with two different weights was present in the conditioned medium from both HUVECs and RASMCs (Fig 2). Since the naturally occurring forms of nonglycosylated and glycosylated VEGF$_{165}$ are 19 and 22

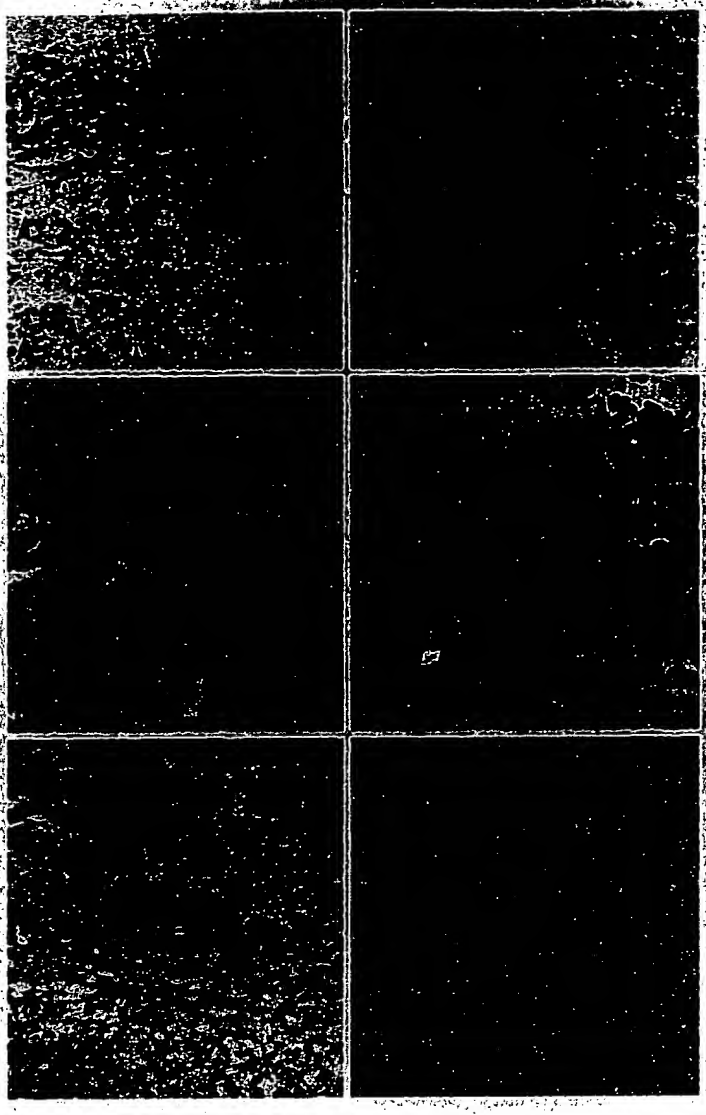

Fig 9. Angiogenesis induced by AdCMV.VEGF$_{165}$ in vivo. Ad vectors (2×10^{10} pfu) were coinjected with 0.5 mL Matrigel, and the gel plugs were evaluated 14 days later. A through D, Coinjection of AdCMV.VEGF$_{165}$ with Matrigel caused a significant increase in vascularity and in the thickness of the stromal matrix surrounding the gel. Arrowheads indicate blood vessels, and red blood cells are present within these vascular structures. E, Coinjection of AdCMV.βgal with Matrigel caused thickening of the stromal matrix but failed to induce angiogenesis in the tissues surrounding the Matrigel. F, Matrigel alone did not induce angiogenesis and did not affect the thickness of the stromal matrix surrounding the gel. Cells present within the Matrigel likely represent fibroblasts, since they did not stain with antibodies against α-smooth muscle actin (vascular smooth muscle cells), against a 160-kD macrophage plasma membrane component (macrophages), or against the integrin $\alpha_v\beta_3$ (endothelial cells) (results not shown). Arrows identify the stroma between the Matrigel plug (mp) and the abdominal muscles. Histological sections stained with Masson's trichrome (original magnification ×400 for panels A, E, and F and ×1000 for panels B, C, and D).

kD, respectively,[19,37] the two bands shown in Fig 3 most likely represent glycosylated and nonglycosylated VEGF$_{165}$.[23,37] This hypothesis is supported by the observation that the VEGF produced by AdCMV.VEGF$_{165}$-infected cells binds to concanavalin A, a lectin that binds glycoproteins (data not shown). In addition, VEGF produced by plasmid transfection exhibits the same pattern.[23] HUVECs and RASMCs that were either not infected or infected with AdCMV.βgal exhibited no VEGF$_{165}$ mRNA (Fig 1), and no VEGF$_{165}$ protein was found in their conditioned medium (Fig 2). This is expected in the case of HUVECs, since they do not normally express VEGF. RASMCs express VEGF[18,19] but apparently at a level far below that obtained when expression is virally driven, since no VEGF was observed during the short exposure times used in these experiments. The expression of VEGF by AdCMV.VEGF$_{165}$-infected HUVECs (20 pfu per cell) was quantified by enzyme-linked immunosorbent assay. The growth factor was already produced 1 day after infection (7 ng · mL^{-1} · 1×10^4 cells^{-1} · 24 h^{-1}). Peak VEGF production was achieved at 3 and 7 days after infection (22 and 21.7 ng · mL^{-1} · 1×10^4 cells^{-1} · 24 h^{-1}, respectively), and it decreased 17 days after infection (6.5 ng · mL^{-1} · 1×10^4 cells^{-1} · 24 h^{-1}). In contrast, no VEGF was found in the conditioned medium from AdCMV.βgal-infected and uninfected HUVECs.

Permeability Assay

To determine whether the VEGF produced after viral infection is functional, we subjected the conditioned media from HUVECs and RASMCs infected with AdCMV.VEGF$_{165}$ to the Miles permeability assay. Both media markedly enhanced vascular permeability, indicating the presence of large amounts of functional VEGF (Fig 3). In contrast, the conditioned media from HUVECs and RASMCs that were infected with AdCMV.βgal or uninfected produced only a minimal response.

HUVEC and RASMC Proliferation

We examined the effect of AdCMV.VEGF$_{165}$ infection on HUVEC and RASMC proliferation in the absence of exogenous mitogens. The two control groups were represented by uninfected cells and cells infected with AdCMV.βgal. HUVEC infection with AdCMV.VEGF$_{165}$ (20 pfu per cell) led to a progressive increase in cell number over the 2-week course of this experiment (Fig 4). In contrast, both control groups exhibited a

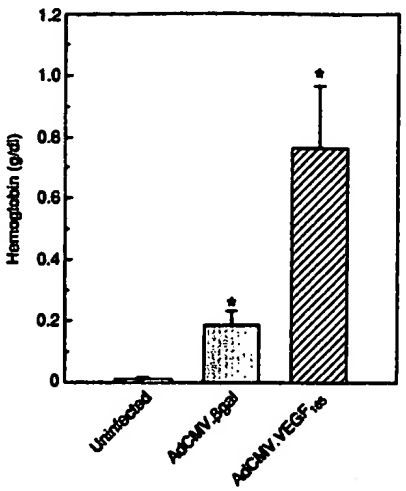

Fig 10. Average hemoglobin content of the Matrigel plugs containing AdCMV.VEGF$_{165}$ and AdCMV.βgal and uninfected control cells. AdCMV.VEGF$_{165}$ caused a significant increase in hemoglobin vs AdCMV.βgal and uninfected control cells, and AdCMV.βgal increased the hemoglobin content vs uninfected control cells. Results represent the average of 10 gel explants for each Ad vector obtained from three separate experiments (*$P \leq .01$ vs either of the other two groups).

progressive decrease in cell number. A different result was obtained with RASMCs studied under conditions otherwise similar to those used for HUVECs. Over the 2-week course of this study, RASMCs infected with AdCMV.VEGF$_{165}$ exhibited a progressive increase in number that was comparable to that observed for the two control groups (Fig 5). Thus, infection of RASMCs with AdCMV.VEGF$_{165}$ did not confer them a growth advantage over the control groups. These results are in agreement with the mitogenic effect of VEGF$_{165}$ being limited to the endothelium.

HUVEC Differentiation

In these experiments, we assessed whether infection with AdCMV.VEGF$_{165}$ could induce differentiation of endothelial cells into capillary-like structures. HUVECs infected with AdCMV.VEGF$_{165}$ and plated on Matrigel under starving conditions were shown to stretch and elongate 4 hours after seeding and formed a stable network by 24 hours (Fig 6). In contrast, HUVECs that were infected with AdCMV.βgal or were uninfected failed to form capillary-like structures.

Ad-Mediated Gene Transfer In Vivo

AdCMV.VEGF$_{165}$ was tested for angiogenesis in vivo by using the Matrigel assay. Initially, we established whether Ad vectors resuspended in Matrigel could diffuse out of the gel and infect the surrounding tissues. For these studies, mice were killed 6 days after injection of Matrigel containing AdCMV.βgal or Matrigel alone, and the Matrigel plugs were stained with X-gal. Fig 7 shows that under these experimental conditions, X-gal–positive cells were found in the stroma surrounding the Matrigel. In contrast, no blue cells were found in the tissue surrounding uninfected gel plugs. In other experiments, the duration of Ad-mediated VEGF$_{165}$ gene expression in vivo was established. By immunohistochemical staining, plugs recovered 3 days after coinjection of Matrigel and AdCMV.VEGF$_{165}$ showed VEGF-positive cells in the tissue surrounding the Matrigel (Fig 8). Staining was most intense at day 7, and only a few cells were immunoreactive 21 days after injection. Incubations in the absence of the primary antibody showed no immunostaining. Incubations with the antibody against the carrier protein showed positivity in the abdominal muscle layer; however, no positivity was found in the tissue surrounding the Matrigel plugs. The Matrigel plugs were examined histologically 14 days after injection, and angiogenesis was observed in the tissues surrounding the Matrigel in response to AdCMV.VEGF$_{165}$ (Fig 9A through 9D). This effect was associated with increased vascularity and thickening of the stromal matrix surrounding the Matrigel. In contrast, AdCMV.βgal resulted in some thickening of the stromal matrix surrounding the Matrigel without evidence of increased vascularization (Fig 9E), and Matrigel alone was not associated with increased stromal thickening or angiogenesis (Fig 9F). Stromal thickness was 28.5±10.4 μm for uninfected plugs (n=8), 104.3±39.2 μm for AdCMV.βgal-infected plugs (n=11, $P \leq .0001$ versus uninfected plugs), and 186.0±46.5 μm for AdCMV.VEGF$_{165}$-infected plugs (n=13, $P \leq .0001$ versus both uninfected and AdCMV.βgal-infected control plugs). Further, the quantitative assessment of angiogenesis (Fig 10) demonstrated that the hemoglobin content of the Matrigel plugs with AdCMV.VEGF$_{165}$ was fourfold higher than in the case of the gel explants with AdCMV.βgal. A significant increase in hemoglobin content was also observed with AdCMV.βgal-infected versus uninfected control plugs. Together, these results show that AdCMV.VEGF$_{165}$ induces angiogenesis in vivo.

Discussion

The present study describes the in vitro and in vivo effects of AdCMV.VEGF$_{165}$, a replication-deficient recombinant Ad vector that carries the cDNA for the human form of VEGF$_{165}$. Both HUVECs and RASMCs infected with AdCMV.VEGF$_{165}$ expressed the mRNA for VEGF$_{165}$ and secreted functional VEGF protein. HUVECs infected with AdCMV.VEGF$_{165}$ proliferated and underwent differentiation in vitro in the absence of exogenous mitogens. In contrast, both uninfected control cells and control cells infected with AdCMV.βgal exhibited a progressive decrease in cell number over the 2-week course of this experiment and failed to form capillary-like structures on Matrigel. The growth-promoting effect of the infection with AdCMV.VEGF$_{165}$ was limited to HUVECs; RASMCs exposed to the Ad vector made VEGF protein but showed no growth advantage over the control groups.

To document whether AdCMV.VEGF$_{165}$ can induce angiogenesis in vivo, the Ad vector was injected subcutaneously in mice with Matrigel used as a vehicle. Under these conditions, the Ad vectors diffused out of the Matrigel and infected the surrounding tissues where transgene expression occurred. After coinjection of Matrigel with AdCMV.VEGF$_{165}$, peak protein production occurred at 1 week, and VEGF was still identifiable in the animals' tissues up to 3 weeks after injection. An angiogenic response was observed and documented histologically by increased vascularity of the tissues surrounding the Matrigel plugs. Furthermore, this effect of

AdCMV.VEGF$_{165}$ was associated with a significant increase in the hemoglobin content of the gel explants versus both infected and uninfected control groups. It has been previously shown that coinjection of angiogenic cytokines with Matrigel elicits neovessel formation within the Matrigel.[36] Under these conditions, endothelial cells as well as other cells invade the Matrigel, and new blood vessels are formed. In contrast, the addition of AdCMV.VEGF$_{165}$ to the Matrigel elicited a neovascular response in the tissues surrounding the Matrigel, because the Ad vector diffused out of the Matrigel and infected cells in the surrounding tissue. It is noteworthy that both AdCMV.VEGF$_{165}$ and AdCMV.βgal increase the thickness of the stroma surrounding the Matrigel. The mechanism for this effect has not been addressed. However, Ad vectors have been shown to cause an inflammatory response in vivo,[38] and inflammatory cells attracted into the tissues under these conditions release mitogens that may be responsible for the increased stromal thickness. It is noteworthy that AdCMV.βgal enhanced the hemoglobin content of the Matrigel plugs versus uninfected control plugs, albeit at a smaller extent than AdCMV.VEGF$_{165}$. There are two possible explanations for this response: (1) inflammation due to the Ad vector may damage preexisting blood vessels and cause leakage of red blood cells into the surrounding tissue, or (2) mitogenic factors released by inflammatory cells have angiogenic properties and may induce neovascularization. However, since no new blood vessels were apparent in response to AdCMV.βgal, it is likely that red blood cell leakage may have been the primary cause for the enhanced hemoglobin content due to AdCMV.βgal.

The role of AdCMV.VEGF$_{165}$ in the treatment of ischemic disorders remains to be determined; however, there is evidence that it may have a therapeutic effect. It has recently been shown that the intra-arterial infusion of VEGF enhances revascularization in a rabbit ischemic hindlimb model[39] and increases collateral blood flow to the ischemic myocardium in dogs.[40] These studies support the concept that therapeutic angiogenesis may become a clinical objective. In this context, gene transfer with a replication-deficient Ad vector may provide the solution to limit exposure to VEGF$_{165}$, in concentrations sufficiently high to induce formation of new blood vessels, only to those tissues in which neovascularization may have a therapeutic effect. Moreover, recent studies have shown that Ad vectors infect cardiac muscle cells when injected directly into the myocardium[10,11,41,42] or into the coronary circulation,[12] and they can also infect skeletal muscle cells.[13] These studies have also shown that foreign gene expression by Ad vectors peaks within the first week after intramyocardial delivery, rapidly decreases thereafter, and is virtually extinguished in 4 to 5 weeks. This apparent limitation of Ad vectors may be advantageous in the case of AdCMV.VEGF$_{165}$, since VEGF$_{165}$ cDNA expression limited to a few weeks and localized to the ischemic tissue may be adequate to induce neovascularization without causing the side effects that may result from prolonged exposure to an angiogenic growth factor.

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